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# POTENTIAL POSTBIOTIC ACTIVITIES OF EXTRACELLULAR PRODUCTS OF PROBIOTIC BACTERIA FROM GILTHEAD SEABREAM GASTROINTESTINAL TRACT

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**Introduction**

Probiotics have been established as a potential tool for improving gut health and environmental quality in aquaculture. However, despite the proven health benefits of probiotics, recent evidence suggests that bacterial viability is not necessary to attain the beneficial-promoting effects (Ang et al. 2020). In this way, postbiotics have emerged providing a potential opportunity in the field of functional foods. They are soluble factors resulting from the metabolic activity of a probiotic or any released molecule, including short-chain fatty acids, enzymes, extracellular products, endo- and exo-polysaccharides, organic acids, etc. that can have interesting properties related to hydrolytic and antagonistic capabilities inducing biological responses on health similar to probiotics while avoiding the necessary administration of live microorganisms (Balthazar et al., 2022). Interestingly, its production can be affected by different factors such as the components of the culture media (Chang et al., 2021) among others. Information on this type of postbiotic activities is very scarce, especially in the case of aquaculture (Mora-Sánchez et al., 2020), so the evaluation of the nutraceutical use of postbiotics to improve health management in fish and other cultivated aquatic organisms is an emerging area of research in aquaculture.

In a previous work, we characterized four potential probiotics (UMA 140, UMA 143, UMA 169 and UMA 216) that were isolated from the gastrointestinal tract of *Sparus aurata* specimens fed with a diet containing a blend of microalgae. This diet involves a selection pressure on the intestinal microbiota of the fish that will be used to achieve enrichment in bacteria with a set of extracellular enzymatic activities capable of metabolizing and mobilizing the components of the diet enriched with microalgae. Here, we investigate the postbiotic potential of the extracellular products (ECPs) obtained from the four candidate probiotics grown on different microalgae-supplemented medium, and evaluate its enzymatic and antibacterial activity, and its cytotoxicity against the SAF-1 cell line. Our objective is to select different ECPs with a variety of activities that help the digestive process of seabream, with the aim to be included in aquafeeds.

**Material and methods**

The four strains (UMA 140, UMA 143, UMA 169 and UMA 216) were grown on tryptic soy agar supplemented with NaCl (1.5 %) (TSAs) at 23º C for 24 h. Then, one to two colonies of each strain were cultured on 50 mL of tryptic soy broth supplemented with NaCl (1.5%) (TSBs) at 23ºC for 36h ($10^9$ UFC $mL^{-1}$, start of the stationary phase) on shaking at 80 rpm. Then, 1 mL of each culture was spread on solid medium plates under the following conditions: i) TSAs (control medium); and solid medium (1.5% agar) supplemented with ii) 5% *Spirulina*; (iii) 5% *Chlorella*; (iv) 5% *Nannochloropsis*; and v) 5% mix of 25% of each algae (*Spirulina*, *Chlorella*, *Nannochloropsis* and *Isochrysis*). *Isochrysis* was not included as a condition because inhibited the culture growth of the four strains. Plates were incubated at 23ºC for 24h and ECPs were obtained by the technique described by Liu (1957). The collection was carried out by adding 2 mL of sterile saline phosphate buffer (PBS). The obtained suspension was centrifuged (10,000 xg, 20 min, 4ºC) and the supernatant was filtered through membranes (0.22µm, pore diameter), and kept at -80ºC until use.

The ECPs were screened for different enzymatic and antibacterial activities. Proteolytic, collagenolytic, lipolytic and amylolytic activities were assayed according to Chabrillon et al. (2005). Phytase, tannase and cellulose hydrolysis were assayed according to Kumar et al. (2010). Antibacterial activity of the ECPs against fish pathogenic bacterial strains *Vibrio harveyi, P. damselae* subsp. *piscicida*, and *Tenacibaculum maritimum* was performed using the agar-well diffusion assay as described by García-Márquez et al. (2021). The hemolytic activity of the ECPs was tested on blood agar plates. In all cases, 50 $\mu$L of ECPs were inoculated into 6 mm-diameter wells made in the plates and incubated at 23ºC for 24-48h. The plates were observed for the presence of a clear zone around the wells. Finally, the absence of cytotoxicity of the ECPs on fibroblast SAF-1 cells from marine gilthead seambream was verified, exposing the cells to different doses of the ECPs. After exposure, the viability of the cells was determined by MTT assay according to Espinosa et al. (2018).

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**Results and discussion**

Only one condition (TSAs 169) was not capable of hydrolyze gelatin, and only two (TSAs 169 and Chlorella 169) did not hydrolyze milk. None of the ECPs had starch, lipase, phytase and tannase hydrolytic activity. Furthermore, only six of the obtained ECPs were capable of hydrolyze the cellulose. In terms of the antibacterial activity of the ECPs, all of them were able to inhibit *P. damselae* subsp. *piscicida*, and only one condition (TSAs 216) inhibited *T. maritimum*.

According to the European Food Safety Authority (EFSA), the evaluation of hemolytic activity is strongly recommended if the isolated bacteria intended to use in food products. In this study, the ECPs obtained from bacterial strains UMA 169 and UMA 216 showed no hemolytic activity. However, ECPs obtained in TSAs medium from strains UMA 140 and UMA 143 showed β-hemolytic activity. Accordingly, due to safety concerns, we discarded from further experiments the ECPs recovered from strains UMA 140 and UMA 143. In view of the results, 5 conditions were selected for further analysis of cytotoxicity: Nanno 169, TSAs 216, Chlorella 216, Nanno 216 and Spirulina 216. The results showed that Spirulina 216 is cytotoxic over the SAF-1 cell line, so its use for addition to feed is not advisable. Further analysis in relation to the contribution of ECP in *in vitro* algae hydrolysis under simulated digestion conditions of seabream enzymes will determine the ECPs candidate with the best potential to be included in aquafeeds.

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